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Rapid fabrication of cell-laden alginate hydrogel 3D structures by micro dip-coating

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Abstract

Development of a simple, straight-forward 3D fabrication method to culture cells in 3D, without relying on any complex fabrication methods, remains a challenge. In this paper, we describe a new technique that allows fabrication of scalable 3D cell-laden hydrogel structures easily, without complex machinery: the technique can be done using only apparatus already available in a typical cell biology laboratory. The fabrication method involves micro dip-coating of cell-laden hydrogels covering the surface of a metal bar, into the cross-linking reagents calcium chloride or barium chloride to form hollow tubular structures. This method can be used to form single layers with thickness ranging from 126 μm to 220 μm or multi-layered tubular structures. This fabrication method uses alginate hydrogel as the primary biomaterial and a secondary biomaterial can be added depending on the desired application. We demonstrate the feasibility of this method, with survival rate over 75% immediately after fabrication and normal responsiveness of cells within these tubular structures using mouse dermal embryonic fibroblast cells and human embryonic kidney 293 cells containing a tetracycline-responsive, red fluorescent protein (tHEK cells).

1. Introduction

Conventional 2D, monolayer cell culture still remains the main approach for the study of cell biology, regenerative medicine and drug discovery [1]. However the relevance of 2D cell cultures, compared to 3D physiological conditions is limited and 3D cell culture approaches are important in narrowing the gap between *in vitro* and *in vivo* studies [2-6]. Current methods for the fabrication of 3D scaffold materials include self-assembly [7], solvent casting [8], dry freezing [9], electrospinning [10, 11] and etc. However, the main barrier in conventional scaffold based tissue engineering approaches is the inability to position living cells precisely to mimic 3D tissue. Repopulation of decellularised tissues and organs has been reported to regenerate 3D tissue and can be used as a platform for drug discovery and organ transplantation, but this approach relies on the availability of donated organs so cannot be scaled up indefinitely [12-14].

3D biofabrication [15-25] is a very promising emerging field that gives experimenters the ability to position cell-laden bio-inks precisely into a pre-designed 3D structure. Recent studies on 3D bioprinted tumour models using Hela cells [4] shows the cells to be more chemo-resistant than in normal 2D monolayer culture, making the 3D system a potentially better model for study of real cancer cells from patient biopsies.

There is, however, a significant problem with current 3D biofabrication approaches: although they can generate simple and precise 3D structures, they rely on specialised bio-printing machinery that is not easily accessible to many cell biologists. Therefore a simple, controllable and straight-forward 3D bio-fabrication method that does not involve complicated machinery such as bioprinting platforms would be very valuable and effective to create biomimetic 3D structures.

In this study we present a new, inexpensive and simple approach to rapid biofabrication that generates cell-laden tubular structures with tuneable micron resolution and the ability to generate multiple-layer hydrogel tubular structures. This approach could potentially be suitable in fabricating microvasculature *in vitro* and other tubular shaped structures within the human body.

Alginate hydrogel was used as the main substrate for this study as it is currently the most widely used biomaterial for 3D biofabrication due to its ease of use, biocompatibility and the control that can be exerted over its biological half-life [15, 26]. To validate this new 3D biofabrication technique, mouse dermal fibroblast cell viability was monitored within the tubular structures over 6 days. Furthermore, tetracycline-inducible gene expression in a human cell line was used to demonstrate that cells within the fabricated tubular structures were alive and responsive to external signals.

2. Materials and methods

2.1. Cell culture

All cell lines used in this study were cultured in 5.0% CO_2 at 37.0 °C. Human embryonic kidney 293 cells containing a tetracycline responsive red fluorescence protein (tHEK cells) were kindly donated by Dr. Elise Cachat (Centre for Integrative Physiology, Edinburgh University, UK) and cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma D5796) with 5 % Fetal Bovine Serum (Biosera, FB-1090/500). Mouse dermal embryonic fibroblasts, kindly donated by Mrs. Audrey Peter (College of Medicine & veterinary Medicine, Edinburgh University, UK), were cultured in Minimum Essential Medium (MEM, Sigma M5650) supplemented with 5% Fetal Bovine Serum and 1% L-Glutamine (Invitrogen, 25030-081).

2.2. Materials and regents

In this study, sodium alginate solutions of 6% and 8% w/v (Product number W201502, Sodium Alginate, Sigma-Aldrich, Gillingham, UK) prepared in deionised water and collagen type I 0.4%, (w/v) from rat tail and dissolved in 20 mM acetic acid (Product number C3867, Sigma-Aldrich, Gillingham, UK) were used as scaffold. 100 mM $CaCl_2$ (Calcium chloride dihydrate CAS number: 010035-04-8, purity ≥99%, Sigma-Aldrich, Gillingham, UK) and 55 mM $BaCl_2$ (Barium chloride dihydrate, CAS number 10326-27-9, ≥99.999% trace metals basis, Sigma Aldrich, Gillingham, UK) prepared in deionised water were used as the cross-linking reagents. Metal bars that were used to carry cell experiments were purchased from OK International (TE718150PK, OK International) where the metal part was extracted from the needles.

2.3. Cell-laden hydrogel preparation

Sodium alginate 8% w/v was sterilised by gamma radiation (IBL-637 CIS-BioINternational gamma irradiator, France) with 10 Gy at the rate of 1 Gy/min. For experiments including cells, 0.5 mL of a cell suspension of either tHEk cells or mouse dermal embryonic fibroblast cells, at concentration of 8×10^6 cells/ mL, was mixed with 1 mL of 8% w/v sodium alginate solution to result a 6% v/w sodium alginate solution with cell concentration of 2.67×10^6/ mL respectively. Similarly 0.4% w/v collagen solution was prepared containing 8 % w/v sodium alginate and sterilised by Gamma radiation with 10 Gy at the rate of 1 Gy/min.0.5 mL of each cell type with concentration of 8×10^6/mL were separately added to result a 0.26% w/v collagen and 6% w/v sodium alginate solution at a cell concentration of 2.67×10^6 /mL.

2.4. Alginate hydrogel single layer tubular structure fabrication by micro dip-coating

Stainless steel metal bars were chosen, because of their wettability: bars with a range of diameters (down to 600 μm) were used as the mould for tubular structure fabrication. The metal bars were kept in ethanol overnight, prior to use, to sterilize them. As depicted in Fig.1a, the mould was first dipped into a 6% w/v sodium alginate solution, with or without cells, or dipped into collagen-sodium alginate solution, with or without cells, for about 3 seconds and then removed. A thin layer of the cell-laden hydrogel was left coated on the surface of the metal bar. The metal bar was then dipped into filter-sterilized cross-linking reagents (100 mM $CaCl_2$ or 55mM $BaCl_2$) for 2 minutes to cross-link the sodium alginate of the coated layer shown in (Fig.1b). The cross-linked cell-laden alginate hydrogel was then gently pulled out by tweezers from one end of the metal bar mould, leaving a hollow tubular structure of cross-linked cell-laden hydrogel shown in (Fig.1c). Depending on the diameters of the metal bar mould, hollow tubular structures with different inner diameters down to 600 μm have been fabricated. For biological studies, cell-laden tubular hydrogel structures were fabricated using a metal bar with 1.2 mm outer diameter.

2.5. Alginate hydrogel multiple layer tubular structure fabrication

To fabricate multi-layered tubular structures, the mould was dipped into sodium alginate for 3 seconds and then dipped in to the cross-linking reagent (100 mM $CaCl_2$ or 55 mM $BaCl_2$) for 2 minutes to cross-link and form the first layer of the tubular structure. The remaining residual solution was removed carefully by absorption by sterilised tissue paper. Then the cross-linked layer, still on the mould, was dipped into to the sodium alginate again for 3 seconds to be coated by the second layer of sodium alginate and then dipped into the cross-linking agent (100 mM $CaCl_2$ or 55 mM $BaCl_2$) for 2 minutes to cross-link and form the second layer. This procedure was repeated as many times as necessary to reach the number of layers desired within the tubular structures.

2.6. Flow experiment through cross-linked alginate hydrogel tubular structures

The flow experiment was designed to investigate whether the fabricated alginate hydrogel tubular structures are sealed against liquid flow within the tubular structure. An alginate hydrogel tubular structure made using a 4 mm rod was fabricated followed by the dip-coating protocol. 60 mL of water with a red dye was prepared and loaded into a 60 mL syringe. The syringe was then loaded into a syringe pump and the tubular structure was fitted to the syringe tip. The syringe pump flowrate was set to 20 mL/sec for 3 minutes.

2.7. Viability assay

Tubular structures containing mouse embryonic dermal fibroblasts were fabricated using 1.2 mm diameter rods according to the above protocol and cultured for 6 days. A LIVE/DEAD® Cell Vitality Assay Kit (L34951, Life Technologies) was used according to the manufacturer's

protocol. Briefly, the medium was replaced with fresh medium containing 500 nM c_{12}-resazurin and 10 nM SYTOX Green before incubating at 37 °C, 5% CO_2 for 15 minutes.

2.8. 3D cell Imaging and viability testing of mouse embryonic dermal fibroblast cells

Confocal images of mouse embryonic dermal fibroblast cells were taken on a Nikon A1R FLIM confocal microscope. Z-Stacks (range of 176 µM, 5 µM steps) of each sample were captured using a Nikon A1R FLIM confocal microscope. Images were analysed using Imaris software to investigate the viability of the cells encapsulated within the tube walls fabricated by 1.2 mm mould at day 1, day 3 and day 6 of culture.

2.9. Inducing the tHEk cells with tetracycline and imaging.

Tubular structures containing tHEk cells within the tube walls were fabricated using 1.2 mm rods by the fabrication protocol and incubated for 24 hrs. After 24 hrs of incubation the medium was replaced with fresh medium contacting tetracycline at concentration of 1 µg/mL and then incubated for further 48 hrs. Images of the induced tHEK cells were captured after 72 hours using Zeiss Axiovert Immunofluorescence microscope.

3. Results and discussions

3.1. Biofabrication of alginate hydrogel tubular structures with variable diameters and alginate concentrations

Our strategy for producing cell-laden tubular structures was to dip a stainless steel rod into a suspension of cells in an alginate-based buffer and then to transfer the rod, still coated with a thin layer of this suspension, into a solution of divalent cations that would cross-link the alginate into a hydrogel (Fig 1a). A variation of the strategy was to repeat this process, building up thicker and thicker hydrogels on the rod, before the rod and hydrogels were separated to leave a cell-laden, hydrogel tube.

We began by testing the idea using acellular solutions of alginate. Tubular structures were successfully fabricated using 6% w/v alginate and stainless steel rods of inner diameters ranging from 0.6 mm, 1.2 mm, 2.5 mm, 3 mm, 4 mm to 6 mm (Fig. 2b), using either 100 mM $CaCl_2$ or 55 mM $BaCl_2$ solution to promote the cross-linking procedure. The thickness of the tube walls formed on rods of 0.6 mm, 1.2 mm, 2.5 mm, 3 mm, 4 mm and 6 mm were 126 ± 6 µm, 143 ± 5 µm, 171 ± 9 µm, 181.6 ± 13 µm, 206.6 ± 6 µm and 220 ± 7 µm respectively (the ± figures indicating standard deviation). These results indicated an interesting correlation between the tube wall thickness and the inner diameter of the tube, where a metal rod (mould) with greater diameter used during fabrication resulted in a larger tube thickness.

To characterise further the relationship between rod diameter, alginate concentration and resulting wall thickness, solutions of 5%, 6%, 7% and 8% w/v alginate were used to fabricate tubular structures. The measurements (Fig. 3) indicated that wall thickness increased approximately linearly with respect to sodium alginate concentration. 5 % w/v alginate was the minimum concentration capable of fabricating tubular structures in this system: tubes made with lower concentrations had insufficient rigidity to maintain the integrity of the structure when the rod was removed. It should be noted that this minimum is probably specific to the sodium alginate used in this study and that other sodium alginates with different viscosities and molecular weights may require different alginate concentrations and different concentrations of cross-linking reagents to fabricate the tubular structures which may or may not result in a tubular structure with either smaller or bigger wall thickness.

The idea of repeating the dip-then-crosslink cycle to build and create thicker walls was tested by transferring cross-linked hydrogel, still on the rod, back into the alginate solution and cross-linking again. This procedure, done 1-3 times, successfully fabricated thicker walls. The distinct layers are visible in transverse sections of the tubes (Fig. 4). Potentially this approach could position different cell types within each layer with the ability to fine-tune each layer to the desired thickness depending upon the application. However, the tube wall thickness after the micro-coating is dependent on the wettability of the coating surface. Therefore the wall thickness of the secondary layer would depend on the wettability of the first coated layer and hence the thickness of the second layer may vary with different batches of material from the data presented here.

In addition as shown in Fig. 5**,** the tubular structure is completely liquid tight from the start to the end of the flow and provided a safe and sealed environment for liquid flow through the tubular structure. The fabricated cell-laden tubular structures here with tuneable diameters could be used as a disease model to mimic and study the flow behaviour and flow-cell interactions inside blood vessels.

3.2. Cell viability in alginate and collagen-alginate hydrogels

Having demonstrated the basic tube fabrication technique using acellular alginate, we went on to produce cell-laden tubes. Tubular structures containing mouse dermal fibroblast cells were fabricated using the dip coating method described above, but with NIH-3T3 mouse fibroblasts suspended in the alginate solution before the rod was dipped in it. In some experiments, collagen I was added to the alginate. Two different cross-linking solutions were tried; $BaCl_2$ and $CaCl_2$. Cells were stained using a LIVE/DEAD® Cell Vitality Assay, which stains living cells red and dead cells green and allowed viability to be monitored through 6 days of culture for each condition. In all conditions, cells could clearly be detected within the tube walls (Fig. 6). All conditions showed good cell viability. Cells within alginate hydrogel tubes and collagen-alginate cross-linked with 55 mM $BaCl_2$ had the highest viability of 84 ± 2.4% and 81 ± 7.4%. Cell viability in alginate hydrogel, and alginate-collagen, cross-linked by $CaCl_2$ was 78 ± 3.1 % and 72 ± 4.4 % (Fig.7). The results showed a good viability after 6 days of culture within the tubular structures for all conditions indicating good biocompatibility of the gel. However, a slight decrease in viability in collagen-alginate mixture cross-linked by 100

mM $CaCl_2$ and 55 mM $BaCl_2$ was observed and was probably due to lower pH level of the collagen solution creating a harsher environment for the cells.

3.3. Cell proliferation

Confocal images of the mouse dermal fibroblast cells were taken for the four different conditions of fabrication at day 1, day 3 and day 6 of culture as shown in figure 6. Three random areas within each tubular structure was selected at 300 μm × 300 μm × 50 μm to count the cell numbers through Imaris software. Based on the results shown in figure 8 for all four conditions cells have grown and proliferated in some parts of the gel and have formed small clusters within the fabricated tube walls. The following results indicated that the fabricated gel has a good permeability and porosity allowing nutrition and oxygen to penetrate into the gel as well as allowing the waste to be extracted from the gel. These optimum conditions created a suitable enough extracellular matrix for cells to grow while supporting cell to cell interaction in some parts of the gel. Figure 6 summarises the cell density within the tubular structures fabricated in four different conditions throughout 6 days in culture. Cell density is considerably higher in alginate cross-linked with $BaCl_2$ where initially also cell viability results indicated highest viability was achieved in alginate cross-linked with $BaCl_2$.

3.4. Responsiveness of cells to small molecules

To validate whether the walls of the tube allow cells to access small signalling molecules, and whether they show normal gene expression responses to these, we used a tHEk cell line that activates expression of Red Fluorescent Protein (RFP) in response to tetracycline [27-29]. The tHEK cells were cultured for 24 hrs within the fabricated tube walls, again made using the four alternative methods of tube manufacture (with and without collagen, and using either $CaCl_2$ or $BaCl_2$ to cross-link). They were then cultured for a further 48 hrs in the presence or absence of tetracycline. Cells cultured with no tetracycline could be detected but showed no red fluorescence (Fig 9a, c,e,g,i). Cells exposed to tetracycline showed a robust induction of RFP expression in all four conditions (Fig 9b, d, f, h and j).

4. Conclusion

In this study we have developed a new rapid 3D biofabrication technique for making alginate hydrogel tubular structures. Importantly, this technique can be done in a standard biology lab and requires no cell sheet technologies [30-33] or other complex tubular fabrication approaches that require expensive and complicated machinery systems [34-41]. Tubular structures from sub-millimetre, or a few hundred micron range, to greater diameters with an ability to control the thickness of the tube walls can be fabricated using the micro dip-coating technique. This

approach might incorporate other bio-materials within alginate hydrogel to help cell biologists to bypass lengthy, complicated and expensive fabrication approaches. They can then use the dip-coating method to fabricate 3D tubular structures with living cells in their preferred extracellular matrix. The fabrication method is gentle to live cells while maintaining high cell viability over 6 days within the tubular structures cross-linked by $CaCl_2$ and $BaCl_2$. It also leaves cells free to interact with small molecules such as the tetracycline used as a demonstration here. This method presents a promising potential for fabricating tubular structures which are better models of anatomy than 2D cultures are, and may be suitable for a range of tubular tissues including embryonic kidney, lymph vessels, blood vessels, trachea and intestine.

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References

[1] Gies K, Kaufmann J, Pronk G J, Klippel A 2002 Unravelling novel intracellular pathways in cell-based assays *Drug Discovery Today* 7(3):179-186.

[2] Imamura Y, Mukohara T, Shimono Y, Funakoshi Y, Chayahara N, Toyoda M, Minami H. 2015 Comparison of 2D- and 3D-culture models as drug-testing platforms in breast cancer *Oncology Reports* 33:1837-1843

[3] Birgersdotter A, Sandberg R, Ernberg I 2005 Gene expression perturbation *in vitro*—A growing case for three-dimensional (3D) culture systems *Seminars in Cancer Biology* 15(5): 405-412

[4] Zhao Y, Yao R, Ouyang L, Ding H, Zhang T, Zhang K, Cheng S, Sun W 2014 Three-dimensional printing of Hela cells for cervical tumor model in vitro *Biofabrication* **6** 035001

[5] Edmondson R, Broglie J J, Adcock A F, Yang L 2014 Three-Dimensional Cell Culture Systems and Their Applications in Drug Discovery and Cell-Based Biosensors *Assay and Drug Development Technologies* 12 (4): 207–218.

[6] Bellis A D, Bernabé B P, Weiss M S, Shin S, Weng S, Broadbelt L J, Shea LD 2013 Dynamic transcription factor activity profiling in 2D and 3D cell cultures *Biotechnology and Bioengineering* 110: 563–572

[7] *Peck M, Dusserre N, McAllister TN, L'Heureux N. Tissue engineering by self-assembly. Mater Today. 2011;14:218–224.*

[8] Thadavirul, Napapaphat, Prasit Pavasant, and Pitt Supaphol. "Development of polycaprolactone porous scaffolds by combining solvent casting, particulate leaching, and polymer leaching techniques for bone tissue engineering." *Journal of Biomedical Materials Research Part A* 102.10 (2014): 3379-3392.

[9] Offeddu G S, Ashworth J C, Cameron R E, Oyen M L 2015 Multi-scale mechanical response of freeze-dried collagen scaffolds for tissue engineering applications. *Journal of the Mechanical Behavior of Biomedical Materials* 42:19-25.

[10] Chu X, Xu Q, Fenf J, Li Q, Sun X, Cao Y, Ding Y 2014 In vitro biocompatibility of polypyrrole/PLGA conductive nanofiber scaffold with cultured rat hepatocytes *Materials Research Express* 1(3):035402

[11] Bini T B, Gao S J, Tan T C, Wang S, Lim A, Hai L B, Ramakrishna S 2004 Electrospun poly(L-lactide- co -glycolide) biodegradable polymer nanofibre tubes for peripheral nerve regeneration *Nanotechnology* 15(11): 1459

[12] L'Heureux N, Paquet S, Labbe R, Germain L, Auger F A 1998 A completely biological tissue-engineered human blood vessel *FASEB* J 12 47–56

[13] Crapo P M, Gilbert T W, Badylak SF 2011 An overview of tissue and whole organ decellularization processes *Biomaterials* 32(12):3233-3243

[14] Wilson S L, Sidney L E, Dunphy S E, Rose J B, Hopkinson A 2013 Keeping an Eye on Decellularized Corneas: A Review of Methods, Characterization and Applications *Journal of Functional Biomaterials 4*(3):114–161

[15] Ghanizadeh Tabriz A, Hermida A H, Leslie N R, Shu W 2015 Three-dimensional bioprinting of complex cell laden alginate hydrogel structures. *Biofabrication* 7(4) 045012.

[16] Faulkner-Jones A, Greenhough S, King J A, Gardner J, Courtney A, Shu W 2013 Development of a valve-based cell printer for the formation of human embryonic stem cell spheroid aggregates. *Biofabrication* 5 015013

[17] Li C, Faulkner-Jones, A, Dun A R, Jin J, Chen P, Xing Y, Yang Z, Li Z, Shu W, Liu D, Duncan R R 2015 Rapid Formation of a Supramolecular Polypeptide–DNA Hydrogel for In Situ Three-Dimensional Multilayer Bioprinting *Angew. Chem. Int. Ed* 54: 3957–3961.

[18] Faulkner-Jones A, Fyfe C, Cornelissen D J, Gardner J, King J, Courtney A, Shu W 2015 Bioprinting of human pluripotent stem cells and their directed differentiation into hepatocyte-like cells for the generation of mini-livers in 3D *Biofabrication* 7 044102

[19] Gudapati H, Yan J, Huang Y, Chrisey D B 2014 Alginate gelation-induced cell death during laser-assisted cell printing. *Biofabrication* 6:035022

[20] Yamaguchi S, Ueno A, Akiyama Y and Morishima K 2012 Cell patterning through inkjet printing of one cell per droplet *Biofabrication* **4** 045005

[21] Kang K H, Hockaday L A, Butcher J T 2013 Quantitative optimization of solid freeform deposition of aqueous hydrogels *Biofabrication* 5(3):035001

[22] Hochleitner G, Jungst T, Brown T D, Hahn K, Moseke C, Jakob F 2015 Additive manufacturing of scaffolds with sub-micron filaments via melt electrospinning writing. *Biofabrication* 2015;7:035002

[23] Ouyang L, Yao R, Mao S, Chen X, Na J, Sun W. 2015. Three-dimensional bioprinting of embryonic stem cells directs highly uniform embryoid body formation *Biofabrication* **7**:044101

[24] Groll J, Boland T, Blunk T, Burdick J A, Cho D W, Dalton P D, Derby B, Forgacs G, Li Q, Mironov V A, Moroni L, Nakamura M, Shu W, Takeuchi S, Vozzi G,Woodfield T B, Xu T, Yoo J J, Malda J 2016 Reappraising the definition of an evolving field *Biofabrication* 8(1): 013001

[25] Kang H W, Lee S J, Ko I K, Kengla C, Yoo J J, Atala A 2016 A 3D bioprinting system to produce human-scale tissue constructs with structural integrity. *Nature Biotechnology* 34(3):312-319

[26] Mørch Ý A, Donati I, Strand B L 2006 Effect of Ca2+, Ba2+, and Sr2+ on Alginate Microbeads *Biomacromolecules* 7(5):1471-1480

[27] Wong H L, Wang M X, Cheung P T, Yao K M, Chan B P 2007 A 3D collagen microsphere culture system for GDNF-secreting HEK293 cells with enhanced protein productivity *Biomaterials* 28(35):5369-5380

[28] Cachat E, Liu W, Martin K C, Yuan X, Yin H, Hohenstein P, Davies J A 2016 2- and 3-dimensional synthetic large-scale *de novo* patterning by mammalian cells through phase separation *Scientific Reports 6*:20664

[29] Ouyang L, Yao R, Chen X, Na J and Sun W 2015 3D printing of HEK 293FT cell-laden hydrogel into macroporous constructs with high cell viability and normal biological functions *Biofabrication* 7:015010

[30] Sekine H, Shimizu T, Yang J, Kobayashi E, Okano T 2006 Pulsatile Myocardial Tubes Fabricated With Cell Sheet Engineering *Circulation 114(1 suppl):* I-87-I-93.

[31] Othman R, Morris G E, Shah D H, Hall S, Hall G, Wells K, Shakesheff K M, Dixon E J 2015 An automated fabrication strategy to create patterned tubular architectures at cell and tissue scales. *Biofabrication* 7(2): 025003

[32] Matsuda, N, Shimizu T, Yamato M, Okano T 2007 Tissue Engineering Based on Cell Sheet Technology *Adv. Mater* 19: 3089–3099

[33] Kubo H, Shimizu T, Yamato M, Fujimoto T, Okano T 2007 Creation of myocardial tubes using cardiomyocyte sheets and an in vitro cell sheet-wrapping device. *Biomaterials* 28(24):3508-3516

[34] Wang A, Ao Q, Cao W, Zhao C, Gong Y, Zhao N, Zhang X 2005 Fiber-based chitosan tubular scaffolds for soft tissue engineering: fabrication and in vitro evaluation *Tsinghua Science and Technology* 10(4):449–453

[35] Yuan B, Jin Y, Sun Y, Wang D, Sun J, Wang Z, Zhang W, Jiang X 2012 A Strategy for Depositing Different Types of Cells in Three Dimensions to Mimic Tubular Structures in Tissues. *Adv. Mater* 24:890–896

[36] Sakai S, Liu Y, Mah E J, Taya M 2013 Horseradish peroxidase/catalase-mediated cell-laden alginate-based hydrogel tube production in two-phase coaxial flow of aqueous solutions for filament-like tissues fabrication *Biofabrication* **5** 015012

[37] Johnson L M, DeForest C A, Pendurti A, Anseth K S, Bowman C N 2010 Formation of Three-Dimensional Hydrogel Multilayers Using Enzyme-Mediated Redox Chain Initiation *ACS Applied Materials & Interfaces* 2(7):1963-1972

[38] Sher P, Oliveira S M, Borges J, Mano J F 2015 Assembly of cell-laden hydrogel fiber into non-liquefied and liquefied 3D spiral constructs by perfusion-based layer-by-layer technique *Biofabrication* **7** 011001

[39] Hume P S, Bowman C N, Anseth K S 2011 Functionalized PEG hydrogels through reactive dip-coating for the formation of immunoactive barriers. *Biomaterials*, *32*(26), 6204–6212

[40] Onoe H, Okitsu T, Itou A, Kato-Negishi M, Gojo R, Kiriya D, Sato K, Miura S, Iwanaga S, Kuribayashi-Shigetomi K, Matsunaga Y T, Shimoyama Y, Takeuchi S 2013 Metre-long cell-laden microfibres exhibit tissue morphologies and functions *Nat Mater* 12(6):584-590

[41] Takei T, Sakai S, Ono T, Ijima H, Kawakami K 2006 Fabrication of endothelialized tube in collagen gel as starting point for self-developing capillary-like network to construct three-dimensional organs in vitro. *Biotechnology and Bioengineering* 95:1–7

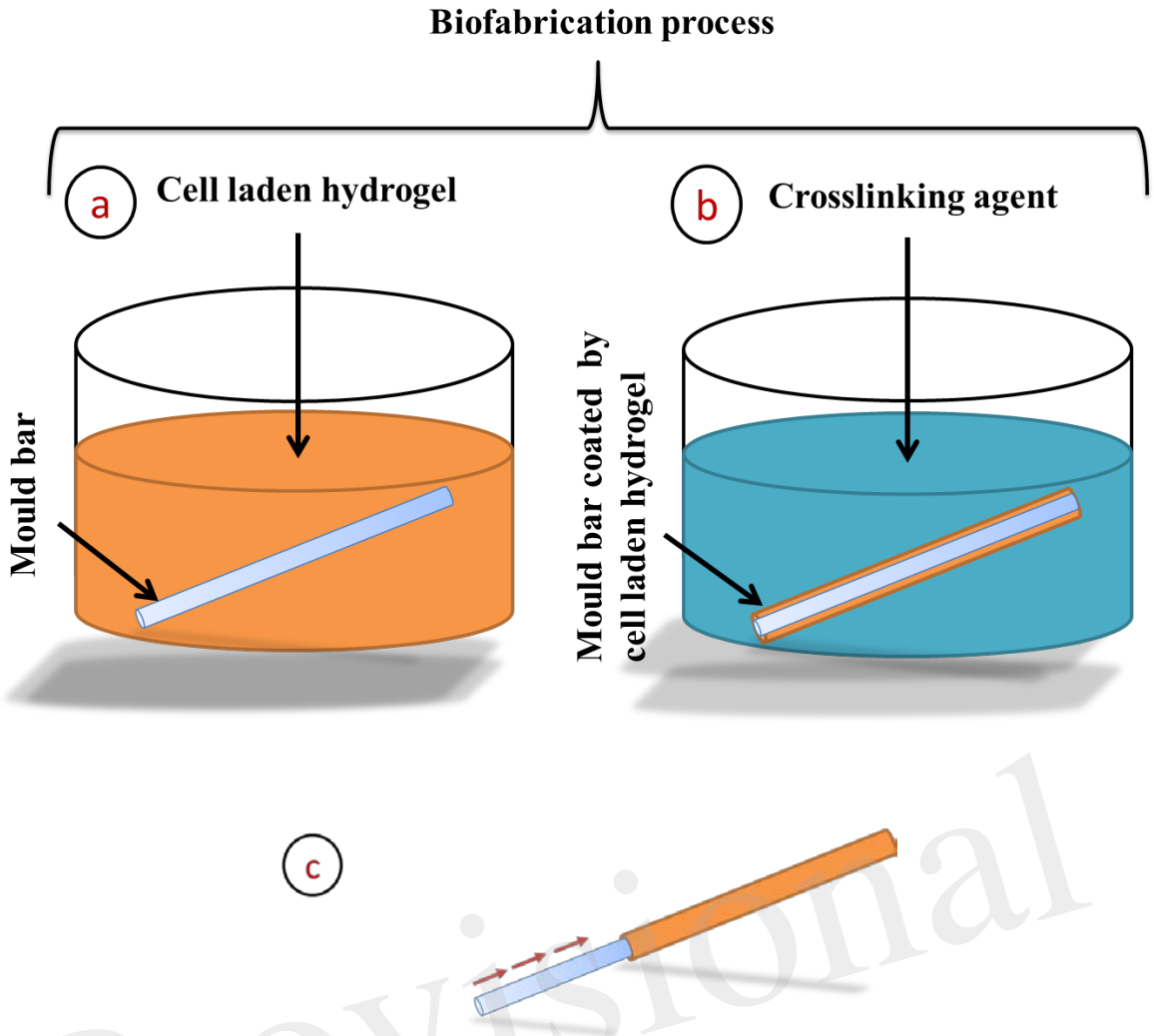

Figure 1. Schematic drawing of the alginate hydrogel tubular structure fabrication process, (a) a metal bar mould is dipped into 6% w/v sodium alginate to coat the surface by a thin layer of cell-laden alginate hydrogel, followed by (b) the exposure to 55 mM $BaCl_2$ or 100mM $CaCl_2$ to fully cross-link the sodium alginate layer for 2 minutes and then (c) the cross-linked alginate layer will be pulled out from the mould as a hollow tube.

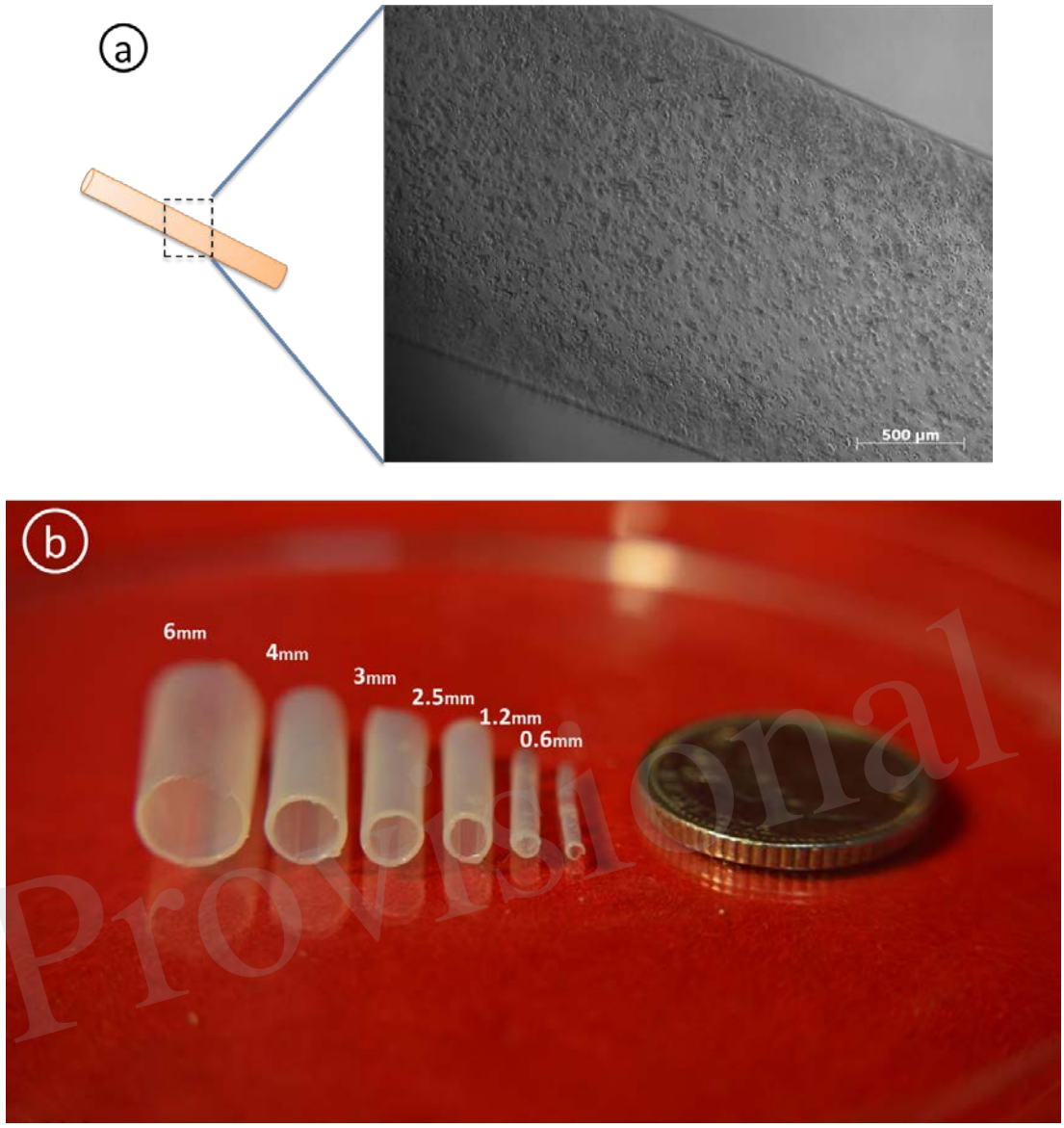

Figure 2. Images of tubes made from alginate. (a) A cell-laden tubular alginate hydrogel structure fabricated via dip-coating (b) Alginate hydrogel tubular structures fabricated by the dip-coating method. Fabricated single layer alginate hydrogel tubular structures with various diameters with descending diameters from left to right. Scale bar: 5p

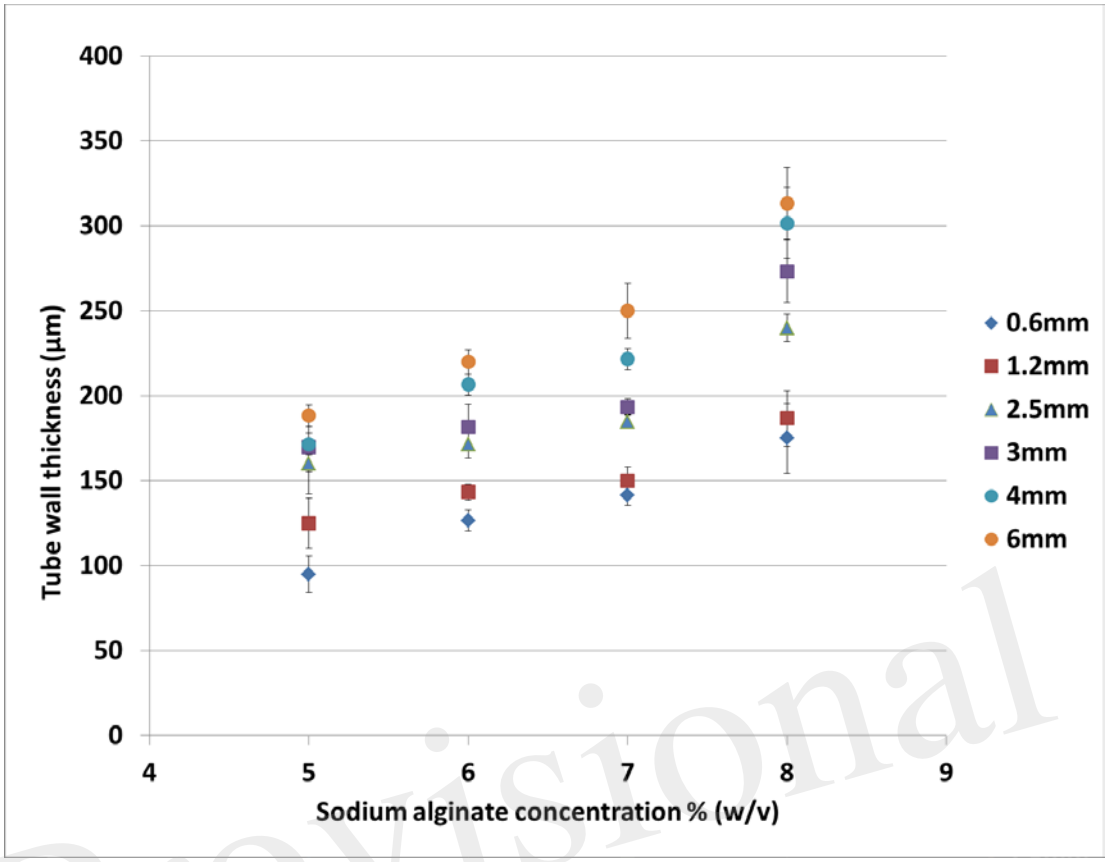

Figure 3. Effect of sodium alginate concentration and rod diameter on the wall thickness of tubes fabricated using one dip in the alginate solution. The rod diameters are indicated to the right of the graph.

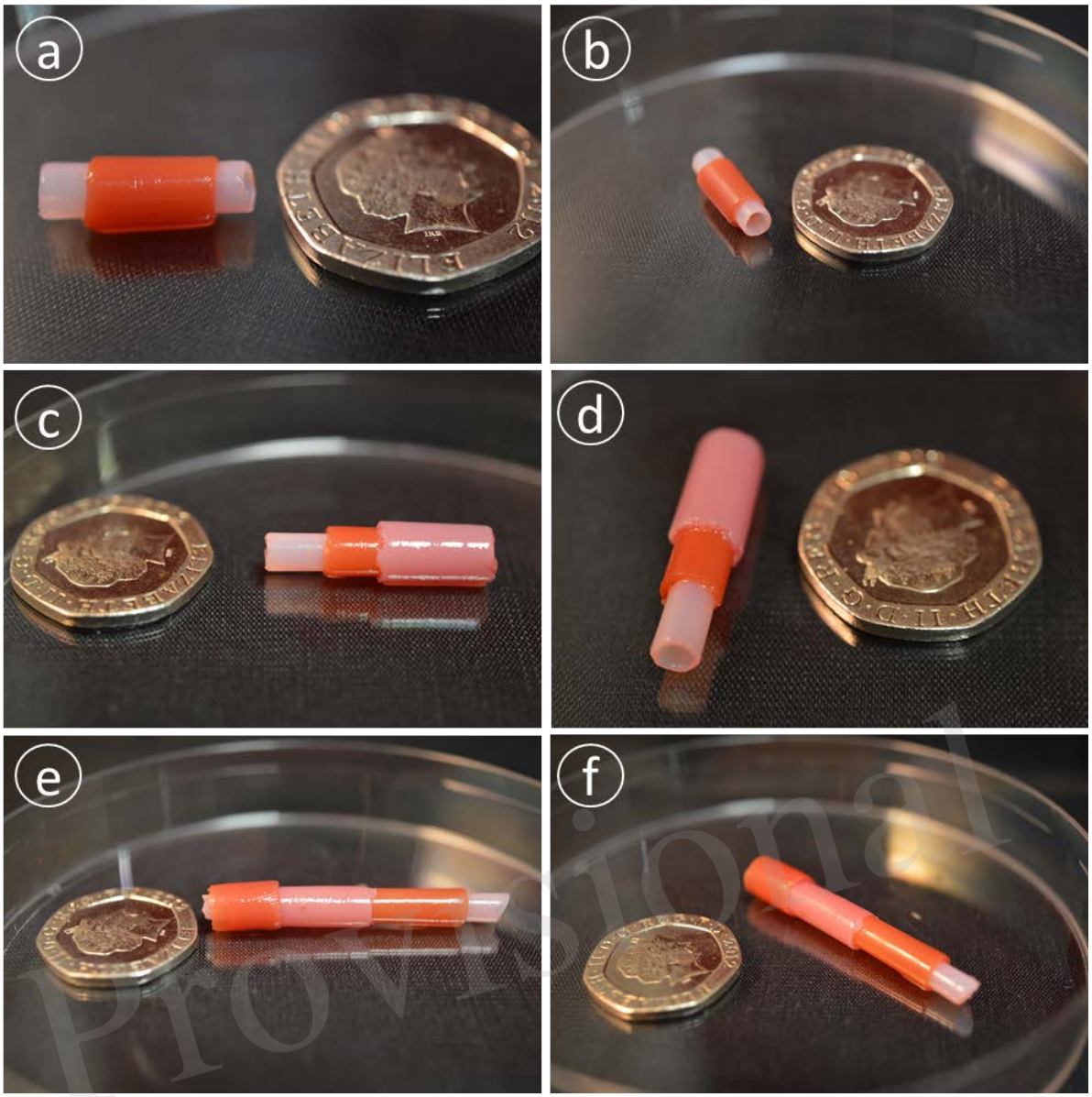

Figure 4. Multi-layer alginate structures. (a and b) show two layers, (c and d) three layers and (e and f) four layers. Each layer was distinguished by making it with an alginate solution with a different dyes. Scale bar: 20p coin.

Figure 5. Flow experiment through a single layer alginate hydrogel tubular structure. The red dye is pumped form a syringe, through the fabricated tube to the beaker on the right. The dye is transferred and does not leak either into water (the beaker on the left) or air (note the absence of dye on the table).

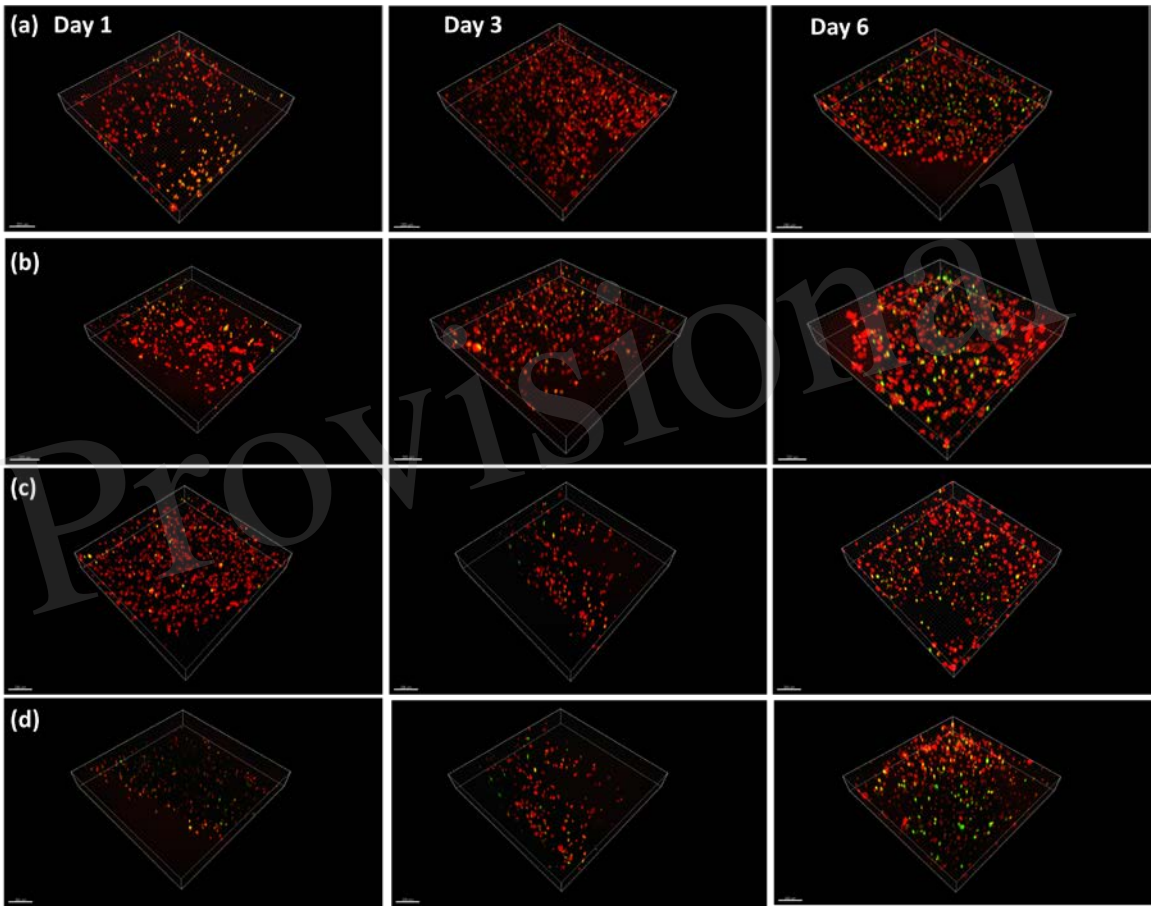

Figure 6. Mouse fibroblast cell lines cultured within the tube walls of (a) alginate hydrogel cross-linked by calcium, (b) alginate hydrogel cross-linked by barium, (c) alginate-collagen cross-linked by calcium and (d) alginate-collagen cross-linked barium over 6 days. Red stain indicates live cells and green stain indicates dead cells. Scale bar: 100 μm.

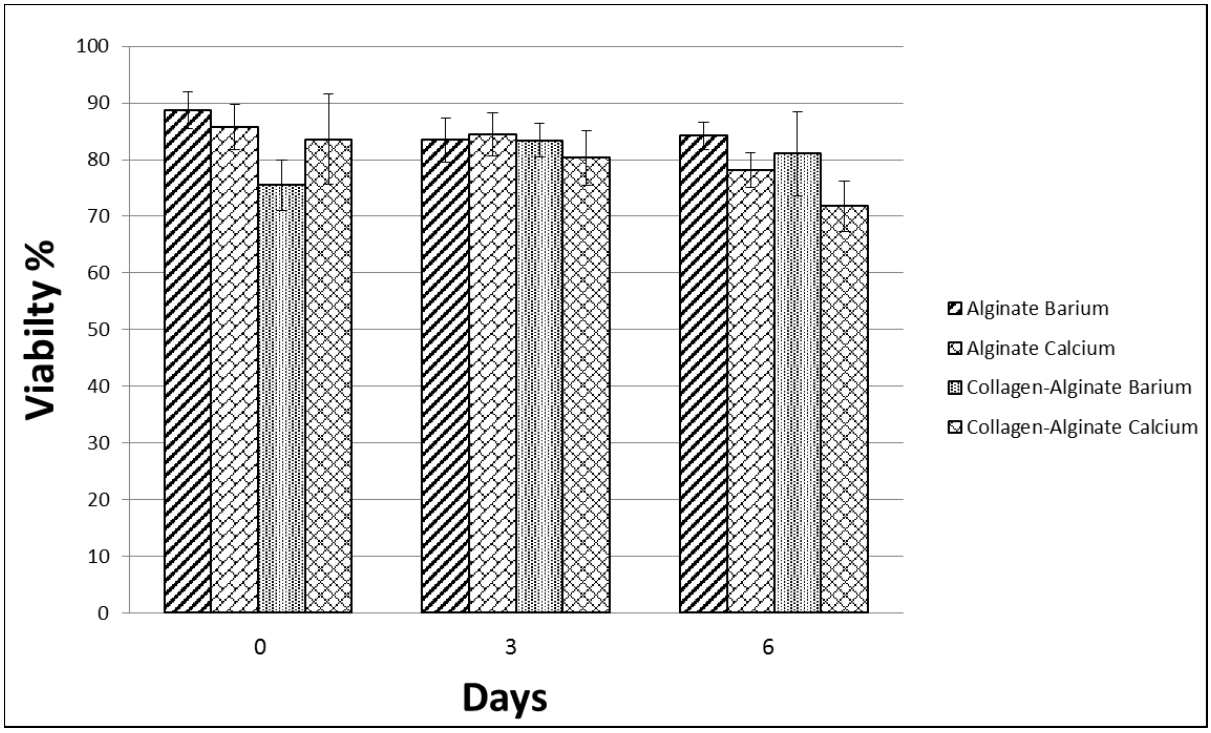

Figure 7. Viability of mouse embryonic dermal fibroblast cells within the tube walls over 6 days of culture fabricated using alginate and collagen-alginate cross-linked by 100 mM $CaCl_2$ and 55 mM $BaCl_2$

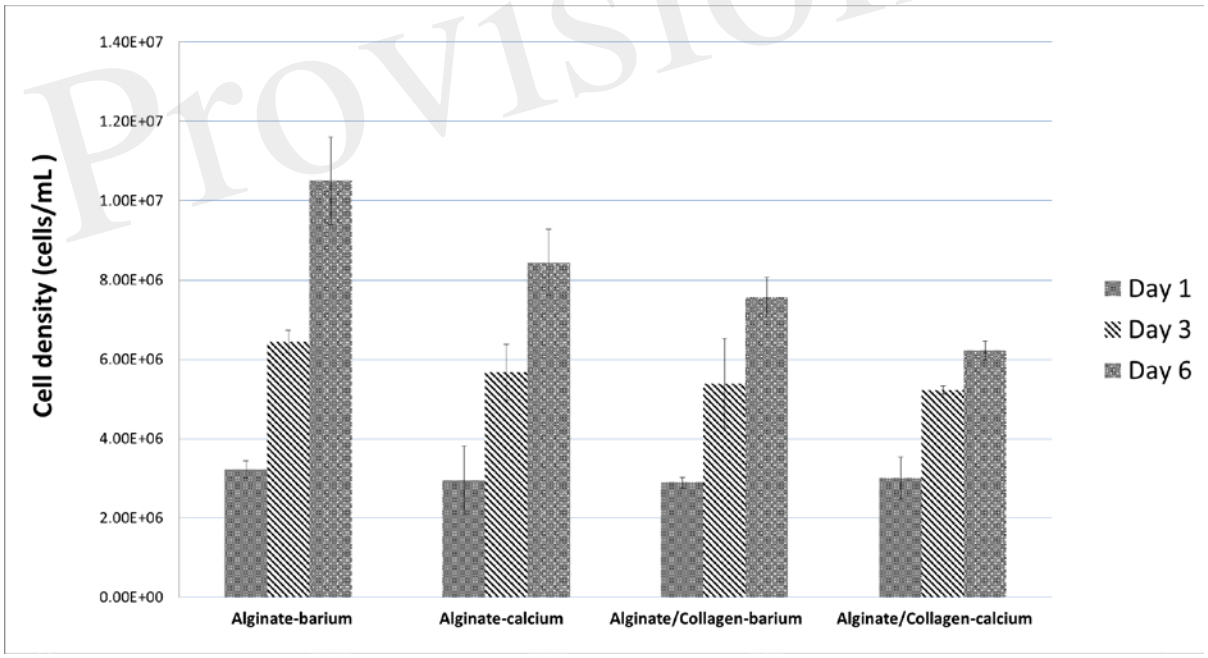

Figure 8. Fibroblast cell numbers within the tubular structures fabricated by four different conditions at day 1, day3 and day 6 of culture.

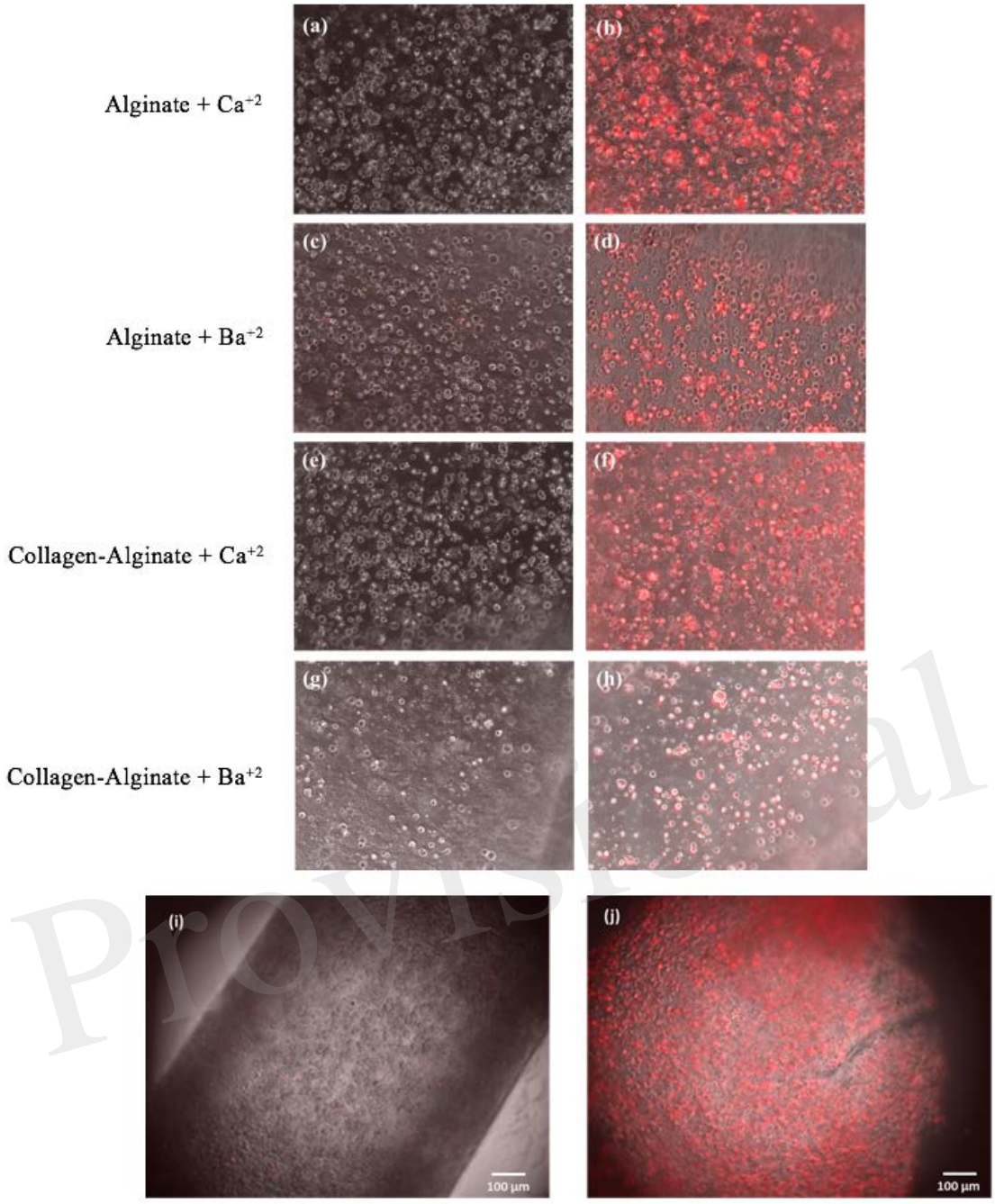

Figure 9. Responsiveness of cells in the wall to small signalling molecules. (a, c, e and g) show tHek cells cultured in tubes made from, and cross-linked by, the reagents indicated, but not exposed to tetracycline. (b, d, f and h) show tHek cells in tubes made the same way, but then exposed to tetracycline after 24 hrs and cultured for a further 48 hrs. Red fluorescent protein is robustly induced. (i) and (j) show low-magnification images of the tubes containing cells before and after induction with tetracycline.